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The response of foodborne pathogens to osmotic and desiccation stresses in the food chain

Burgess, C M ; Gianotti, A ; Gruzdev, N ; Holah, J ; Knøchel, S ; Lehner, A ; Margas, E ; Schmitz Esser, S ; Sela (Saldinger), S ; Tresse, O

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Review

The response of foodborne pathogens to osmotic and desiccation stresses in the food chain



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ABSTRACT

In combination with other strategies, hyperosmolarity and desiccation are frequently used by the food processing industry as a means to prevent bacterial proliferation, and particularly that of foodborne pathogens, in food products. However, it is increasingly observed that bacteria, including human pathogens, encode mechanisms to survive and withstand these stresses. This review provides an overview of the mechanisms employed by *Salmonella* spp., Shiga toxin producing *E. coli*, *Cronobacter* spp., *Listeria monocytogenes* and *Campylobacter* spp. to tolerate osmotic and desiccation stresses and identifies gaps in knowledge which need to be addressed to ensure the safety of low water activity and desiccated food products.

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1. Introduction

In the food industry, salt, in combination with other “mild technologies” is often used as a general preservative and an antibacterial agent because of its inhibitory effects on bacterial growth in ready-to-eat

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(RTE) meat, seafood, and fermented foods such as salami, cheese, baked goods, fruit and vegetables (Desmond, 2006). In addition, salt is often considered an essential additive to enhance the flavour, texture and shelf life of meat products (Ruusunen and Puolanne, 2005). Salt can cause damage to bacterial cells by disrupting the maintenance of osmotic balance between the cytoplasmic and intracellular environments (Csonka, 1989). Hyperosmotic solutions of sugars have been used for the dehydration and reformulation of ready to eat fruits (Torreggiani and Bertolo, 2004). Osmotic dehydration treatment has also been adopted as a partial dewatering process by immersion of fruit and vegetable tissues in hypertonic solutions (Rahman, 2008). Osmotic dehydration represents a mild process to improve the fresh-cut product stability and quality, along with other preservation technologies (i.e. sanitation, refrigeration, modified atmosphere packaging (Torreggiani and Bertolo, 2004)). Besides the diffusion of water from the vegetable tissue simultaneous solutes' counter-diffusion into the tissue is usually observed (Kowalska and Lenart, 2001) and the sucrose concentration seems to cause a hindrance of microbial cell adhesion to fruit surfaces (Gianotti et al., 2001).

Bacteria may encounter osmotic stress during a shift to a hyperosmotic solution or due to dehydration. Changes in osmolarity pose significant stress on bacterial cells by causing either swelling in hypotonic environments or dehydration and shrinkage under hypertonic environments (Csonka, 1989; Sleator and Hill, 2002). The term water potential represents the work involved in moving 1 mol of water from some point in a system (at constant pressure and temperature) to a pool of pure water at atmospheric pressure and at the same temperature as the system under consideration, while matric water potential generally is applied to considerations of water interactions at surfaces and interfaces (Abee and Wouters, 1999; Potts, 1994). When water molecules are associated with interfaces (including foods) such as the surfaces of colloidal particles (solid particles that range from ~ 0.002 to $1\ \mu\text{m}$ in diameter, e.g., proteins, ribosomes, some bacteria, and viruses) in an aqueous solution, they have less tendency to react chemically in bulk solution or to escape to the surrounding vapour phase. Interfaces thus lower the thermodynamic activity of the water, especially near the surface of the colloid (Potts, 1994). Interfaces together with solutes lower the water activity (a_w), so that there is an additive effect in solutions containing solutes and colloids.

Moreover, water efflux occurs when bacterial cells are exposed to a gas phase with an a_w that is lower than the cell compartment. If there is a considerable difference between the water activities of the two compartments, exposure of the cells for a limited time may lead to rapid shrinkage of the cytoplasm. However, if the a_w of the gas phase is sufficient to permit growth, albeit slow growth, the cells may achieve a water balance through de novo synthesis of compatible solutes.

The removal of a substantial fraction of the bulk water from cells through a drying stress is termed desiccation, and such desiccation can be achieved through either rapid or slow drying. There is one fundamental distinction between matric and osmotic systems: the immediate environment of a cell under desiccation (matric stress) is the atmosphere; i.e., the surfaces of their cell walls are exposed to a gas phase, while cells under osmotic stress are bathed in an aqueous solution, albeit in one of diminished a_w (Potts, 1994).

The question of desiccation also represents a challenge for food preservation, surface disinfection, pathogen transmission and, at the same time, an opportunity for the production of probiotics and dried cultures for the dairy, beer and wine industries. Regarding the former it is important to diminish microbial viability as efficiently as possible, while for the latter viability needs to be kept high (Nocker et al., 2012). Shrinkage of the cell's capsular layers, increase in intracellular salt concentrations and macromolecules due to a decrease in cell volume are the main consequences of desiccation (Potts, 1994). Other effects include changes in biophysical properties (such as surface tension), reduced fluidity of membrane lipids, and damage to proteins and DNA. Moreover, one of the molecular mechanisms of damage leading to death in desiccation-

sensitive cells upon drying is free-radical attack to phospholipids, DNA and proteins. Regulation of the antioxidant defence system is complex and its role in desiccation tolerance is not yet firmly established, although in cyanobacteria cells of *Nostoc commune*, adapted to intense solar irradiation, Fe-superoxide dismutase was demonstrated to be the third most abundant solute (Shirkey et al., 2000). In general, as recently confirmed by Nocker et al. (2012), Gram-negative species are greatly more susceptible to drying than the Gram-positive species. The reasons for the higher resistance of Gram-positive bacteria are thought to be related to their smoother surfaces, the thicker peptidoglycan layer and the lack of lipopolysaccharides (Miyamoto-Shinohara et al., 2008).

Disaccharides and extracellular polysaccharides show a clearly protective effect against desiccation. In particular trehalose and sucrose, form 'supersaturated solid solutions' (such as glasses) with high viscosity, that form when the sugar solution becomes highly concentrated due to water loss (Koster, 1991). The benefits of such glasses for cells undergoing desiccation include filling space to prevent cellular collapse and continuance of hydrogen bonding at the interface between the glass and the cells (Koster, 1991). Membrane lipids, protected by the formation of hydrogen bonds between the sugar and the phospholipid head groups, contribute to the maintenance of normal lipid structure in the membranes (Welsh and Herbert, 1999). Similar effects apply to proteins (Leslie et al., 1995).

It is well known that the presence of extracellular polysaccharides in biofilms protects cells from desiccation and other stresses. Potts (1994) described that the shrink-swell behaviour of extracellular polysaccharides under conditions of different water potentials affected the pore sizes and passage of solutes. The authors hypothesised that the low permeability of extracellular polysaccharides results in a 'hydraulic decoupling' during rapid wetting or drying events and therefore effectively shields extracellular polysaccharide-embedded cells from adverse effects of extreme fluctuations in hydrated conditions. Additionally, Nocker et al. (2012) demonstrated that magnesium chloride concentrations $\geq 50\ \text{mM}$ dramatically increase bacterial susceptibility to desiccation in the case of Gram-negative bacteria, and to a lesser extent also for Gram-positive bacteria.

The ability to survive and/or proliferate under stresses such as osmotic and desiccation stress is well known to contribute to the persistence of pathogens both in foods and food-processing environments, elevating the risk of transmission of pathogens through the food chain to humans. However, a further aspect sometimes overlooked is the possibility that exposure to osmotic stress along the food chain may lead to cross-protection against subsequent stresses faced in food production or during transit in the GI tract. For example, some mechanisms known to contribute to osmotic stress resistance, such as compatible solute transporters and cold shock proteins, can contribute to *L. monocytogenes*' ability to grow at low temperature (Sleator et al., 2003). It has also been demonstrated by phenotypic data that growth at low temperature provides cross-protection to subsequent salt stress (Bergholz et al., 2012) and that at $37\ ^\circ\text{C}$, exposure of *L. monocytogenes* to osmotic stress increases its resistance to subsequent exposure to bile salts (Begley et al., 2002). Another example is represented by the accumulation of compatible solutes such as glycine betaine and carnitine, the mechanism used by bacterial cells to overcome osmotic stress, which mainly stabilises enzymes and proteins, thus ensuring their continuous function in adverse conditions. It was demonstrated by Jørgensen et al. (1995) that the heat resistance of *L. monocytogenes* increased with the time it had been exposed to salt in a rich medium. Heating in 9% NaCl compared with a medium without added NaCl resulted in an 8-fold increase in heat resistance, while growth for 48 h and heating in the same medium gave a 22-fold increase. Furthermore, *Listeria* requires osmolyte uptake systems to maintain the full bile tolerance in vitro and the presence of carnitine contributes significantly to bile tolerance (Watson et al., 2009).

It is clear that bacteria, including foodborne pathogens, have evolved a number of complex interplaying systems to tolerate desiccation and

osmotic stress. This is of significant concern to the food industry which relies on the use of low a_w and desiccation to ensure the safety and quality of their products. Therefore, it is vitally important that there is a clear understanding of how individual pathogens respond and adapt to these environmental stresses, to enable the development of appropriate risk reduction measures. In this review, the quantitative response to osmotic/desiccation stresses and the molecular mechanisms underlying this response will be examined for five key foodborne pathogens, namely, *Salmonella*, verocytotoxigenic *Escherichia coli*, *Cronobacter*, *L. monocytogenes* and *Campylobacter* to osmotic and desiccation stress. While the confirmed human cases of salmonellosis are steadily falling in Europe, cases of campylobacteriosis are stationary and are increasing for listeriosis and VTEC infections (EFSA, 2015).

2. Non-typhoidal *Salmonella* spp.

Non-typhoidal *Salmonella* include more than 2500 serovars belonging to the species *Salmonella enterica*, with some of them common foodborne pathogens, responsible for morbidity and mortality worldwide, as well as for significant economic losses to the food industry. International and national outbreaks of salmonellosis linked to consumption of contaminated food are frequently reported (Beuchat et al., 2013). *Salmonella* may colonise the intestinal tracts of host animals, and consequently, most outbreaks of salmonellosis result from consumption of contaminated foods of animal origin, such as meat, eggs and poultry. Nonetheless, the presence of *Salmonella* has been reported in numerous low-moisture foods, such as whole egg powder, dried herbs, seed, spices, soy bean meal, tahini, minced dehydrated white onions, peanut butter, black pepper, dried mushrooms, oat cereals, low a_w sausage, chocolate and flour. Indeed, multiple large national and international outbreaks have been linked to consumption of low a_w foods (Table 1), suggesting that this pathogen can survive in low a_w food items throughout their often long shelf-life until the point of consumption. The high virulence of this pathogen, combined with its high environmental persistence, make *Salmonella* a major challenge for the food industry (Humphrey, 2004).

Exposure of *Salmonella* to osmotic or desiccation stress results in the loss of water that causes considerable shrinkage of the microbial cell due to water loss (Csonka, 1989), with a consequent increase in concentrations of all the intracellular metabolites. Sudden plasmolysis may result in the inhibition of a variety of physiological processes, ranging from nutrient uptake to DNA replication (Csonka, 1989).

In order to maintain turgor pressure, the cells respond by the induction of an array of adaptive physiological responses aimed at increasing the concentrations of compatible solutes and osmoprotectants. In *Salmonella* these include potassium ions (Measures, 1975), glutamate, glutamine, proline (Csonka, 1981; Csonka, 1982), glycine-betaine (Le Rudulier and Bouillard, 1983), or choline or trehalose (Gjaever et al., 1988). There is a hierarchy of osmotic solutes in their utilisation and effectiveness; *Salmonella* prefer betaine over proline-betaine which, in turn, is preferred over proline (Csonka and Epstein, 1996). The initial response of bacteria to hyperosmotic shock is an increase of K^+ uptake. Two main transport systems are responsible for this function: Trk, a constitutive low-affinity system and the rapidly induced Kdp system, which has a high affinity to K^+ (Spector and Kenyon, 2012). The Trk system consists of three components: TrkA (peripheral membrane protein), TrkE (membrane associated), and TrkG (membrane-spanning protein), and is expressed constitutively. The Kdp system has very high affinity for K^+ , its expression highly depends on the osmolarity of the medium (Csonka and Epstein, 1996) and it is also composed of three parts: KdpA (membrane-spanning protein), KdpB (integral membrane protein), and KdpC (peripheral membrane protein). In order to maintain electroneutrality, the cell next increases the glutamate pool by glutamate synthesis. This process is catalysed by two enzymes: glutamate dehydrogenase and glutamate synthase (Csonka, 1989).

In ongoing hyperosmotic stress, trehalose synthesis is induced by the products of the *otsAB* operon (Csonka and Epstein, 1996), and the levels of K^+ /glutamate start to decline. Trehalose production is dependent on the alternative sigma factor, RpoS for the induction of *otsAB* (Kempf and Bremer, 1998). RpoS expression is known to be induced during osmotic shock (Rychlik and Barrow, 2005). If osmoprotectant molecules, such as glycine-betaine, proline, or choline, are present externally, they may be taken into the cell by specific transporters, such as ProP (glycine-betaine, proline), ProU (glycine-betaine) and OsmU (betaine; (Jovanovich et al., 1988)). DNA supercoiling has also been suggested to be important in the induction of ProU (Higgins et al., 1988).

After initiating the described mechanisms of osmoprotectants' uptake or synthesis, cells which are placed in a hyperosmotic environment can start passive diffusion of osmoprotectants (Balaji et al., 2005). *S. Typhimurium* has two major porins which function as channels to facilitate the diffusion of small hydrophilic molecules: OmpF and OmpC. High osmolarity causes a decrease of OmpF and an increase of OmpC levels (Feng et al., 2003). This is mainly due to the smaller size of the OmpC porin which blocks larger molecules, allowing only smaller ones to come through. The transcription of *ompC* is mediated by a two-component regulatory system involving a membrane sensor protein, EnvZ, which activates the signal-transducing regulatory protein, OmpR, in response to periplasmic signals (Feng et al., 2003).

Under desiccation, a portion of the *Salmonella* population may change into a physiologically dormant state described as viable but nonculturable (VBNC) (Gruzdev et al., 2012b; Lesn et al., 2000; Podolak et al., 2010). Transcriptome analyses of *S. Enteritidis* in peanut oil demonstrated that bacteria entered into a physiologically dormant state, with <5% of its genome being transcribed, compared to 78% in a nutrient-rich medium (Deng et al., 2012). This phenomenon may result in an under-estimation of *Salmonella* contamination in low-moisture foods and consequently poses a challenge to food safety testing.

Mattick et al. (2000a) observed the formation of filaments when *Salmonella* cells were placed in hyperosmotic solutions ($a_w = 0.92$ – 0.96); however Eriksson de Rezende et al. (2001), found filamentation at an a_w of 0.95 but not at 0.92. Although the two studies were performed with different settings and strains, it seems that filamentation is probably not a common mechanism for *Salmonella* persistence during dehydration and long-term storage (Gruzdev and Sela, unpublished results).

Another response to low a_w environments that has been reported in *S. Enteritidis* cells, is the degradation of ribosomal RNA molecules (Deng et al., 2012), which might serve as a potential source of nutrients required for the adaptation of the bacterium to the low a_w environment. This finding is in agreement with the need for increased biosynthesis of osmoprotectants and an observed increase in fatty acid catabolism (Finn et al., 2013b; Li et al., 2012).

Additional changes in cell metabolism upon exposure to low a_w environments involve the cell membrane. An increase in the ratio of *trans* to *cis* unsaturated fatty acids has been observed in cells exposed to high salt concentrations (Cronan, 2002), and the proportion of anionic phospholipid and/or glycolipids was also increased (Russell et al., 1995).

Salmonella can survive for long durations on dry surfaces and in foods (Table 2). Margas et al. (2014) assessed the survival of 15 isolates of *Salmonella* dried onto stainless steel surfaces over 30 days. For most of the strains, following an initial reduction in viability in the first hours (<72 h), no further reduction was seen and a 2-population Weibull model was fitted to model the survival kinetics. While there was a significant difference in the strains desiccation survival, for the most resistant strains, the model indicated that time was an insignificant resistance factor. With respect to survival factors, Hiramatsu et al. (2005) reported that the survival rates of *Salmonella* in dry foods and in a desiccation model system were significantly increased by the presence of sucrose. Similarly, dehydration of *Salmonella* in the presence of the compatible solutes, such as trehalose and sucrose, facilitated both

Table 1Examples of outbreaks of foodborne *Salmonella* infections associated with consumption of contaminated dry foods.

Year	Food product	People affected/ country	Serotype	Possible cause/comments	References
2013	Tahini Sesame Paste	16, USA	Montevideo Mbandaka	FDA inspection identified: failure to store raw material to avoid contamination, failure to protect food transported by conveyor from contamination, not adequate plumbing and drainage	CDC (2013)
2012	Peanut butter	42, USA	Bredeney	FDA inspection identified: failure to manufacture foods under conditions and controls necessary to minimise potential growth and survival of microorganisms (e.g. failure with cleaning and disinfection, lack of appropriate hygienic design of the equipment and factory, water presence on surfaces, lack of appropriate segregation). Positive samples from the environment were obtained.	FDA (2013)
2011	Turkish pine nuts	43, USA	Enteritidis	Bulk bags contaminated (no information available about the source)	CDC (2011)
2010	Salami	272, USA	Montevideo	Black and red crushed pepper imported from Vietnam was a source of contamination; it was added after the lethality step.	CDC (2010)
2008	Cereal	28, USA	Agona	Presumably dust from the disruption of the previously intact wall (contaminated with <i>Salmonella</i> from 1998 outbreak) during plant maintenance activities, in combination with wet cleaning, may have reintroduced the desiccated outbreak strain of <i>Salmonella</i> Agona into the cereal production area.	Russo et al. (2013)
	Peanut butter	684, USA, Canada	Typhimurium	Route of contamination not known	CDC (2009)
	Powdered infant formula	8, France	Give	Route of contamination not known	Jourdan et al. (2008)
2007	Snack (rice-corn)	65, USA	Wandsworth Typhimurium	The seasoning mix used in Veggie Booty may be the source of the contamination,	CDC (2007b)
2006	Peanut butter	715, USA	Tennessee	<i>Salmonella</i> was isolated from environmental samples from the manufacturing plant and environmental contamination is likely source of the outbreak. It was suggested that the contamination was persistent for prolonged period of time.	CDC (2007a), Sheth et al. (2011)
	Chocolate	37, UK	Montevideo	Leaking pipe has been implicated as a source of contamination. Waste water was dripping into the chocolate crumb.	Anon (2006)
2005	Almonds	15, Sweden	Enteritidis	Almonds were not treated	Muller et al. (2007)
	Cake mix	26, USA	Typhimurium	Route of contamination not known	Zhang et al. (2007)
2004	Powdered infant formula	141, France		The source of contamination in the manufacturing plant was not established, however a former and persistent environmental contamination was suspected.	Cahill et al. (2008)
2003	Almonds	29, USA, Canada	Enteritidis PT9c	Almonds were not treated. One environmental sample at the manufacturer premises and three samples from huller-shellers were positive for <i>Salmonella</i>	CDC (2004)
	Tea (aniseed)	42, Germany	Agona	Batch of tea was contaminated with aniseed cultivated in Turkey that have been fertilised with manure	Koch et al. (2005)
2002	Tahini	68, Australia, New Zeland	Montevideo	Exact contamination route not known. It is suspected that sesame seeds were contaminated during harvesting	Unicomb et al. (2005)
2001	Chocolate	439, Denmark, AT, DE, Belgium, Finland	Oranienburg	Contaminated chocolate was produced during one week. Source was never determined. Hygienic violations were not observed in the factory. Milled cocoa beans received additional heat-steam treatment with 125–130°C. It is not clear if <i>Salmonella</i> survived heating process or contamination was introduced after.	Werber et al. (2005)
	Halva	70, AU, DE Norway, Sweden, UK	Typhimurium DT104	Contamination was associated with sesame seeds produced in Turkey	de Jong et al. (2001)
	Peanuts	109, Australia, Canada, UK	Stanley, Newport	Asian-style peanuts were implicated as a source, some of them were roasted some of them just dried and flavoured.	Kirk et al. (2004)
2000	Almonds	168, USA, Canada	Enteritidis PT30	<i>Salmonella</i> was isolated from raw almonds, factory equipment (6–7 months after being used), warehouse, orchards and distribution. It was recovered for prolonged period on frequent basis which suggest persistent contamination.	Beuchat et al. (2013)
	Powdered infant formula	30, Korea	London	It was not possible to determine whether the PIF was contaminated during manufacture or after opening of the package for consumption, because the organism was only found in an opened package of formula.	Cahill et al. (2008)
1999	Coconut	18, UK	Java PT Dundee	Route of contamination not known	Ward et al. (1999)
1988	Cereal	209, USA	Agona	Route of contamination not known	CDC (1998)
1996	Infant dried milk	12, UK, France	Anatum	<i>Salmonella</i> was not found in environmental samples from the production plant. The product samples which were positive for <i>Salmonella</i> were produced from the same batch of raw ingredients	Investigation internationale: Belgique (1997)
	Peanut butter	54, Australia	Mbandaka	Route of contamination not known	Beuchat et al. (2013)
	Powdered infant formula	>48, Spain	Virchow	<i>Salmonella</i> was isolated from the milk powder however there were no positive samples from the factory environment. Exact route of contamination is not known	Usera et al. (1996)
1995	Infant food	5, UK	Senftenberg	Cleaning remains from milling machinery were implicated as a source. The machinery was used to mill other products which may have not been heat treated.	Rushdy et al. (1998)
1994	Snack (savoury)	2200, UK, USA, Israel	Agona PT15	S Agona was not isolated from any of the swabs taken from the production line, ingredients, or stool samples from workers. S. Enteritidis was isolated from a reusable plastic bag in which the snack was stored for 48 h before packaging. Contaminated batches were produced on at least 7 separate dates during four months.	Killalea et al. (1996), Shohat et al. (1996)
1993	Potato chips	1000, Germany	Saintpaul, Rubislaw, Javiana	Contaminated paprika powder from South America was the source. The powder was applied to the roasted chips in the end of the production when the temperature dropped to 60°C	Lehmacher et al. (1995)
	Powdered infant formula	3, Canada, USA	Tennessee	FDA isolated S. Tennessee from production equipment in the plant were the formula was dried	CDC (1993)
1989	Snack (corn)	47, UK	Manchester	Three <i>Salmonella</i> serotypes (including S. Manchester) were isolated from site producing yeast extract used for flavouring. These serotypes were also found in stream water used for cooling processing water in the factory.	Beuchat et al. (2013)

Table 1 (continued)

Year	Food product	People affected/ country	Serotype	Possible cause/comments	References
1987	Chocolate	361, Norway, Finland	Typhimurium	<i>S. Typhimurium</i> was not isolated from 11 different raw products and dry ingredients and environmental dust and debris from the factory, however different <i>Salmonella</i> serovars were isolated from environmental dust. Birds could access the plant and introduce contamination. Inspection of the factory revealed several routes of possible contamination of the product.	Kapperud et al. (1990)
1985	Chocolate Infant dried milk product	33, USA, Canada 76, UK	Nima Ealing	Route of contamination not known The source of infection was traced to the factory spray-drier, which had a hole in its inner lining, allowing escape of powder and its return from contaminated insulation material.	Hockin et al. (1989) Rowe et al. (1987)
1982 1981	Chocolate Pepper (black)	245, UK 126, Norway	Napoli Oranienburg	Route of contamination not known Contaminated pepper was shipped from Brazil and it was not treated	Gill et al. (1983) Gustavsen and Breen (1984)
1973	Chocolate,	95, USA, Canada	Eastbourne	Cocoa beans were most likely the source of contamination. The Centre for Disease Control (U.S.A.) found <i>S. Eastbourne</i> in roasted cocoa beans, in rooms dedicated to bean cleaning and roasting and on the inside of a tempering tank. The inspection revealed numerous opportunities for cross contamination in the plant and also identified a number of deficiencies in the chocolate processing operations.	D'Aoust et al. (1975)
	Powdered milk	3000, Trinidad	Derby	Workers may have been vehicles of <i>Salmonella</i> Derby.	Beuchat et al. (2013)
1972	Fishmeal	17, USA	Agona	Contamination spread from fishmeal to poultry and further to restaurant kitchen. The same strain was isolated from shrimps.	Beuchat et al. (2013)
1970	Cocoa	109, Sweden	Durham	Cocoa was contaminated and manufacturing process was not sufficient to kill possible <i>Salmonella</i> contamination.	Beuchat et al. (2013)

dehydration tolerance and subsequent persistence during cold-storage (Gruzdev et al., 2012b). Recent studies on air-dried *Salmonella* Tennessee desiccated on sterile quartz demonstrated a massive accumulation of trehalose following exposure to desiccation (Li et al., 2012). The roles of the extracellular polysaccharide matrix and O-antigen capsule in long-term survival and persistence of *Salmonella* in dry conditions have been studied by a number of authors (Garmiri et al., 2008). White et al. (2006) found that the expression of thin aggregative fimbriae (Tafi) and cellulose enhanced the resistance of *Salmonella* to desiccation on dry plastic surfaces in the absence of nutrients. In contrast, Garmiri et al. (2008) observed no difference in *Salmonella* survival in dried blood droplets in a knockout mutant of the cellulose synthetase gene. Recently, Finn et al. (2013b) demonstrated the importance of the ABC transporter, ProP in desiccation tolerance of *Salmonella* on stainless steel. Another protein which was found to play a critical role in desiccation tolerance is the pathogenicity-related factor SEp22 (Tamura et al., 2009). As SEp22 was found to be identical to Dps, a ferritin-like DNA-binding protein that protects DNA during oxidative stress (Halsey et al., 2004; Martinez and Kolter, 1997), it was suggested that SEp22

protective activity is related to stabilisation of DNA during dehydration and re-hydration as well as to protection against oxidative stress (Amano, 2011; Tamura et al., 2009). Recently, both the DNA-binding activity and the ferroxidase activity, which neutralises and sequesters potentially damaging chemical species, were demonstrated to contribute to protection of *E. coli* against different stresses, including oxidative stress (Karas et al., 2015).

The mechanisms of *Salmonella* survival in low a_w environments are still not completely understood. However, several recent studies have started to bring new perspectives on the underlying adaptation mechanisms (Deng et al., 2012; Finn et al., 2013a, 2013b; Gruzdev et al., 2012a; Huang et al., 2007; Li et al., 2012). Transcriptomic profiles of *S. Tennessee*, a desiccation tolerant strain, and *S. Typhimurium* LT2, a less tolerant strain, was compared following 2 h air-drying at 11% relative humidity. *S. Typhimurium* exhibited high expression levels of protein biosynthesis genes, while *S. Tennessee* demonstrated an increased expression of genes associated with stress response, envelope modification, trehalose biosynthesis and biodegradation pathways (Li et al., 2012). Up-regulation of ribosomal protein genes was also

Table 2

Survival of *Salmonella* spp. on low a_w surfaces and in food products.

Product/surface	Storage temperature ^a	Maximal Survival period	<i>Salmonella</i> serovar	Reference
Milk chocolate	22 °C	60 weeks	Anatum	Barrile and Cone (1970)
Pecan shells	4 °C	78 weeks	Oranienburg, Enteritidis, Anatum, Tennessee, Sundsvall	Beuchat and Mann (2010)
Peanut butter	5 and 21 °C	24 weeks	Agona, Montevideo, Michigan, Enteritidis, Typhimurium	Burnett et al. (2000)
Peanut butter	4 and 25 °C	4 weeks	Oranienburg, Enteritidis, Anatum, Tennessee, Typhimurium	He et al. (2011)
Paper	4, 25, 37 °C	24 months	Oranienburg, Chester, Enteritidis, Litchfield, Anatum, Virchow, Typhimurium	Hiramatsu et al. (2005)
Polystyrene	4 °C	100 weeks	Typhimurium	Gruzdev et al. (2012b)
Cocoa butter oil, crushed cocoa and hazelnut shells, cocoa beans and almond kernels	5 and 21 °C	21 days	Enteritidis, Napoli, Oranienburg, Poona, Montevideo, Typhimurium	Komitopoulou and Penalzoza (2009)
Halva	4 °C	32 weeks	Enteritidis	Kotzekidou (1998)
Egg-shell	15 and 25 °C	20 days	Enteritidis	Messens et al. (2005)
Pasta		12 months	Typhimurium, Infantis	Rayman et al. (1979)
Chocolate	22 °C	9 months	Typhimurium, Eastbourne	Tamminga et al. (1976)
Sesame seeds	4 and 22 °C	16 weeks	Newport, Montevideo, Typhimurium	Torlak et al. (2013)
Almond kernels	–20, 4, 23 & 35 °C	78 weeks	Enteritidis	Uesugi and Harris (2006)
Dry soil	5 and 22 °C	12 weeks	Typhimurium	Zibilske and Weaver (1978)
Stainless steel	25 °C	>4 weeks	Typhimurium, Agona, Enteritidis, Muenchen, Napoli, Tennessee, Typhimurium DT104, Cubana, Lomita, Mbandaka	Margas et al. (2014)

^a Temperatures in bold indicate the storage temperature with maximal survival time.

observed in air-dried *S. Typhimurium* SL1344 on polystyrene surfaces (Gruzdev et al., 2012a). In addition, Gruzdev et al. (2012a) reported on the induction of genes involved in amino acid metabolism, stress response, energy production and ion transport. Some similar transcriptomic profiles were also reported in a *S. Typhimurium* ST4/74 strain that was dehydrated on stainless steel (Finn et al., 2013b). The latter study demonstrated down-regulation of 187 genes and up-regulation of 79 genes involved in the transport of osmoprotectants, trehalose biosynthesis, formation of iron–sulphur (Fe–S) clusters, histidine metabolism and oxidative stress response (Finn et al., 2013b). The involvement of several of the upregulated genes in desiccation tolerance has been studied by site-directed mutagenesis. These included genes encoding isocitrate lyase, *aceA*, the lipid A biosynthesis palmitoleoyl acyltransferase, *ddg*, the modular Fe–S cluster scaffolding protein, *nifU*, the global regulator gene, *fnr* (Gruzdev et al., 2012a), the alternative sigma factor, *rpoE* (Finn et al., 2013b; Gruzdev et al., 2012a), and several transporters, *proP*, *proU*, *osmU* (Finn et al., 2013b) and *kdpA* (Gruzdev et al., 2012a). Although the transcriptomic studies have shed new light on the genes that take part in *Salmonella*'s response to low a_w environments, the specific role of each gene in the bacterial stress response mechanism, remains to be further elucidated.

Development of cross-tolerance to diverse environmental stresses, following exposure to a single stress is common in bacteria (Abee and Wouters, 1999; Kültz, 2004; Vorob'eva, 2004). Similarly, cross tolerance has also been reported in *Salmonella* following exposure to osmotic- or desiccation stress. It is now established that *Salmonella* present in high osmolality or in low a_w foods or media develop increased thermo-tolerance (Breeuwer et al., 2003; Canovas et al., 2001; Kirby and Davies, 1990). Kirby and Davies (1990) demonstrated that dehydration on hydrophobic membranes for 48 h at 37 °C increased the heat tolerance of *S. Typhimurium* LT2 to heat challenge (135 °C/30 min and 100 °C/1 h). Artificially contaminated peanut butter, a low a_w product, exerted heat tolerance upon *Salmonella* with only 3.2-log reduction at temperatures as high as 90 °C (Shachar and Yaron, 2006). Similarly, *Salmonella* was able to survive at 76 °C for more than ten hours in dry milk powder (McDonough and Hargrove, 1968). Correlation between low a_w and thermo-tolerance has also been observed in wheat flour (Archer et al., 1998), corn flour (VanCauwenberge et al., 1981), chocolate (Barrile and Cone, 1970; Goepfert and Biggie, 1968), cocoa and hazelnut shells (Izurieta and Komitopoulou, 2012), liquid media (Chiewchan et al., 2007), egg products (Garibaldi et al., 1969) and dried milk (McDonough and Hargrove, 1968).

Exposure of *Salmonella* cells to low a_w environments increases tolerance to other stresses including sodium hypochlorite (Hiramatsu et al., 2005; Kieboom et al., 2006), and ethanol (Kieboom et al., 2006). In a recent study, the issue of cross-tolerance of dried *S. Typhimurium* to multiple stresses was studied (Gruzdev et al., 2011). Dehydrated *Salmonella* had developed increased tolerance to nine of ten tested stresses, including high NaCl concentrations, bile salts, UV irradiation, dry heat, ethanol, sodium hypochlorite, didecyl dimethyl ammonium chloride, and hydrogen peroxide. Surprisingly, the desiccated bacteria were more susceptible to organic acids (acetic and citric acid), compared to control (wet) bacteria (Gruzdev et al., 2011).

Non-typhoidal *Salmonella* comprise a large number of pathogenic serotypes which are frequently involved in outbreaks associated with the consumption of low a_w foods. Physiologic-, genetic- and more recently transcriptomic studies have contributed to an initial understanding of how this pathogen responds to hyperosmotic and/or dry environments. The role of compatible solutes in protecting cells against hyperosmotic damage is well established. However, the specific roles of numerous other stress induced genes requires further research. In-depth knowledge regarding the cellular stress-response that leads to bacterial persistence is vital in order to understand the risk of *Salmonella* contamination in food of low moisture and in order to develop control measures to limit the pathogen's survival in the food industry during the processing of low a_w ingredients.

3. Shiga toxin producing *E. coli*

Shiga toxin producing *E. coli* (STEC) are foodborne pathogens of zoonotic origin which are responsible for a number of human gastrointestinal illnesses, ranging from watery or bloody diarrhoea to haemorrhagic colitis and haemolytic uremic syndrome. While most outbreaks have been attributed to *E. coli* O157, increasingly, outbreaks associated with other serogroups including O26, O111, O103, O104 and O145 are being reported. In recent years confirmed cases of non-O157 STEC infection has equalled or surpassed O157 cases in the US and Europe (Bosilevac and Koohmaraie, 2011; EFSA, 2007). STEC have been isolated from a number of different animals but ruminants, and bovines in particular, are considered the pathogen's primary reservoir (Duffy et al., 2014). Infection generally occurs as a result of consumption of contaminated foods of bovine origin, including beef and milk products. However, outbreaks have also been associated with consumption of contaminated fresh produce and water (Chekabab et al., 2013). Infection can also occur as a result of animal to person transmission or person to person transmission. Outbreaks of STEC infection have been linked with dried and low a_w foods, including deer jerky (Keene et al., 1997), dry fermented sausage (Conedera et al., 2007; Paton et al., 1996; Sekse et al., 2009) and in shell hazelnuts (Miller et al., 2012). A key concern for the food industry is the low infectious dose of STEC, and its well reported acid stress response mechanisms. From a food safety perspective it is important to gain a more in-depth understanding of other stresses which may be encountered in the farm to fork chain, including its response to high osmolality and desiccation stress.

Much of the research on *E. coli* response to osmolality stress has focused on laboratory strains and non-STEC isolates. Nonetheless, homologues for many of the reported genes can be identified in the genome of sequenced *E. coli* O157 strains (Hayashi et al., 2001; Perna et al., 2001). In STEC the focus of osmolality studies has predominantly been on *E. coli* O157. However, it has been reported that other STEC serogroups of clinical concern are thought to respond in a similar manner to *E. coli* O157 when exposed to food processing stresses (Smith and Fratamico, 2012). Nonetheless, Strain variation and source is an important consideration when examining osmotic tolerance. In a study comparing isolates temporally or geographically located to the 2006 *E. coli* O157 spinach outbreak the environmental isolates were observed to be the most fit isolates, with spinach-derived isolates showing intermediate survival and clinical isolates least capable of surviving osmotic challenge (Parker et al., 2012).

A common osmotic shock response mechanism is the accumulation of selected solutes such as trehalose, proline, glycine betaine and inorganic ions such as potassium in the cytoplasm. When an osmotic upshift occurs *E. coli* responds by uptaking potassium initially (Wood, 1999) which is accompanied by the synthesis of glutamate (Dinnbier et al., 1988). Similar to *Salmonella*, *E. coli* encodes two potassium uptake systems, Kdp and Trk. The Kdp-ATPase complex is composed of four subunits and its expression is stimulated under potassium limitation and osmotic stress by the KdpD-KdpE two component system (Altendorf et al., 1994). The Trk system is composed of a translocating subunit, a regulatory subunit and an ATP binding cassette (Corratge-Faillie et al., 2010). YggT has been demonstrated to compensate for growth defects lacking these potassium uptake systems and it has been suggested that this occurs by altering metabolic pathways to produce osmolytes (Ito et al., 2009).

Potassium is replaced by compatible solutes for longer term adaptation to osmotic stress. Three different transport systems located in the cytoplasmic membrane contribute to this process. ProP is a compatible solute-H⁺ symporter and is activated by an increase in osmolality of the extracellular medium (Racher et al., 1999) through the activity of two promoters which are also under the control of RpoS (Mellies et al., 1995). ProU is a binding protein dependent transport system which, like ProP, can transport substances such as glycine betaine with high affinity, but also a range of other organic compounds (Lucht and

Bremer, 1994). The BetT transporter imports choline, enabling the intracellular synthesis of glycine betaine and its expression is upregulated by osmotic stress and the presence of choline and oxygen (Eshoo, 1988). The production of trehalose in *E. coli* is encoded by *otsA* and *otsB* whose expression is upregulated by osmotic stress or by RpoS (Hengge-Aronis et al., 1991). Other membrane proteins also play a role in osmoregulation. OmpF and OmpC are porins which allow the diffusion of small hydrophilic molecules and whose expression is differentially controlled by the two component system EnvZ-OmpR (Cai and Inouye, 2002). The presence of certain compatible solutes in the growth media results in metabolic shifts when *E. coli* is grown in the presence of increasing concentrations of NaCl. In the presence of proline, metabolic efficiency decreased with increasing NaCl concentration, but in the presence of choline or glycine betaine, it increased between 2 and 4.5% NaCl before declining at 5% NaCl. In the presence of glycine betaine, between 4.5 and 5% NaCl, *E. coli* metabolism switched from aerobic to fermentative pathways, similar to the response for oxidative stress (Metris et al., 2014).

The role of RpoS, a general stress response regulator, in *E. coli* O157's response to osmotic stress has been demonstrated and it regulates a number of genes associated with osmotic tolerance. A RpoS mutant was shown to survive significantly less than its parent strain in 12% NaCl at various temperatures (Stasic et al., 2012). Furthermore, an *E. coli* study demonstrated that the osmotolerance conferred by RpoS is solute dependant, the NaCl sensitive phenotype of a $\Delta rpoS$ mutant can be addressed by the addition of glycine betaine and stationary phase cells exhibit greater osmotolerance in comparison to cells in the exponential growth phase (Cebrian et al., 2015). An earlier study demonstrated increased activity of the *rpoS* promoter under osmotic stress, but only at 37 °C and room temperature, and not at 5 °C (Gawande and Griffiths, 2005). These authors also observed increased promoter activity for other stress response genes, including *uspA* and *grpE*. A study focused on the impact of in vivo passage of *E. coli* O157 resulted in a strain more sensitive to NaCl, with a corresponding decrease in *rpoS* expression (Asakura et al., 2005). Considering the involvement of *rpoS* in the osmotic stress response, it is perhaps not surprising that cross tolerance has been observed between osmotic stress and other stresses. One study demonstrated that acid adaption enabled one strain of *E. coli* O157 to tolerate higher levels of NaCl, but this protective effect was not observed for another O157 strain. A non-O157 strain used as a control was able to tolerate elevated levels of NaCl following both acid shock and acid adaptation (Garren et al., 1998).

A number of studies have shown the role of colanic acid, which can play a role in biofilm formation, in protecting *E. coli* O157 from osmotic stress. One study showed that cells which were incapable of producing colanic acid had a significantly lower survival in the presence of 1.5 M or 2.5 M NaCl (Chen et al., 2004). In a study examining the transcriptomic and proteomic response of *E. coli* O157 to low temperature and low a_w stress Kocharunchitt et al. (2012) demonstrated an increase in expression of genes associated with the Rcs regulon involved in colonic acid biosynthesis. Correspondingly, they observed the greatest levels of colanic acid synthesis at lower a_w values, albeit noting that an inability to produce colanic acid was not detrimental to the growth or survival of *E. coli* O157 in the conditions tested. This increased expression was also observed following an upshift in osmolarity (Kocharunchitt et al., 2014). The role of cellulose, another exopolysaccharide, in providing osmotic protection for STEC has also been investigated and it was reported that cellulose producing strains were protected in response to osmotic stress in comparison to cellulose deficient strains, although some exceptions were observed (Yoo and Chen, 2010).

Similar to *Salmonella*, *E. coli* and STEC have been shown to form filaments under low a_w conditions. Mattick et al. (2003) demonstrated that a panel of STEC and commensal *E. coli* formed filaments at an a_w of 0.96, as well as under a variety of other conditions such as low and high temperatures and low and high pH. The authors point out that, for a low infectious dose organism such as STEC, this may be of particular concern

because ingestion of even one cell could result in bacterial numbers that exceed the infectious dose, upon septation and division.

Transcriptomic and proteomic studies have provided a more broad view of how *E. coli* O157 responds to osmotic stress. Genes associated with chemotaxis and motility have been shown to be downregulated in *E. coli* O157 in response to hyperosmotic stress (Kocharunchitt et al., 2012). Another study showed that a sudden upshift in osmolarity results in an increase in transporters for compatible solutes, as might be expected (Kocharunchitt et al., 2014). A study focused on genes involved in virulence, namely *epsJ-tccP*, reported there was a decrease in their expression in the presence of 0.3 M NaCl, which was not expected (Garmendia and Frankel, 2005). Using an RNA-Seq transcriptomic analysis approach Zhao et al. (2013) identified a *hha* homologue on the virulence plasmid pO157_Sal which was shown to play a role in NaCl tolerance. With the continuing expansion of these types of studies, as well as the sequencing of non-O157 STEC strains there is great potential to better understand the complex interplay between systems to enable this pathogen to survive osmotic stress.

The ability of STEC to withstand desiccation also needs to be considered in the context of food safety. Few, and not recent, studies have specifically focused on the role played by trehalose in the desiccation tolerance of *E. coli* during both air and freeze drying (Leslie et al., 1995; Welsh and Herbert, 1999). Furthermore, there is no consensus in scientific literature about the protectant role of other compatible solutes. An important contribution to understanding the survival of *E. coli* in desiccation conditions comes from soil based studies, where *E. coli* resists environmental stresses, in particular the soil desiccation stress caused by the natural cycles of soil wetting and drying. *E. coli* O157 has been shown to survive in soil for periods ranging from 25 to 231 days (reviewed by Duffy et al. (2014)). A study focused on non-O157 STEC in a number of soil types observed D values ranging from 31.6 to 75.6 days (Bolton et al., 2011). Trehalose can play a role in such environments; as reported by Zhang and Yan (2012) most of the *E. coli* strains considered in their experiments specifically and significantly synthesised trehalose under desiccation conditions. Moreover, the soil adapted *E. coli* strains, in general, produced more trehalose than the non-soil reference strains.

Whether trehalose is added as an excipient prior to drying, or accumulated as a compatible solute in response to osmotic stress, it has been demonstrated to increase desiccation tolerance. According to Welsh and Herbert (1999) the protective effect of exogenously added trehalose was concentration dependent, up to a threshold value of 350 mM. However, trehalose alone cannot explain the intrinsically greater desiccation tolerance of stationary cells compared to exponential phase *E. coli* cells, although their tolerance was also enhanced by exogenous or endogenously accumulated trehalose.

More recently, Stasic et al. (2012) studied *E. coli* O157:H7 persistence in osmotically challenging environments, especially sterile faeces, for an extended time period and they concluded that it was tolerant to desiccation and increased temperature during desiccation and osmotic stress was detrimental to survival. These authors confirmed that *rpoS* regulated genes, involved in the regulation of acid, heat, salt tolerance and in the synthesis of trehalose, are necessary for persistence during desiccation and osmotic stress conditions.

Inoculation studies of *E. coli* O157 onto walnut kernels demonstrated that the pathogen could be detected for many months, even when inoculated at low levels, indicating its tolerance to dry environments (Blessington et al., 2012). Another study which examined STEC tolerance to desiccation dried the cultures onto paper discs and then stored them at different temperatures (Hiramatsu et al., 2005). At 4 °C the majority of STEC strains (12/15) survived for 22 to 24 months, but at elevated storage temperatures of 25 °C and 35 °C survival was less than 70 days and 35 days respectively. The presence of sucrose substantially increased survival. The authors also demonstrated that the desiccated bacteria were able to survive high temperature exposure of 70 °C for 5 h and that STEC strains were more tolerant than non-

pathogenic *E. coli*. Another study which focused on the survival of STEC air-dried on surfaces at different relative humidities (RH) demonstrated that at 70% RH and 12 °C, strains could be detected up to 19 days and that survival decreased when the temperature was increased to 20 °C. This study also observed that the STEC strains were more resistant to desiccation than non STEC strains and that glucose provided a protective effect (Moretro et al., 2010).

While *E. coli* O157 and non-O157 STEC may not be associated with outbreaks of illness associated with low a_w and dried foods as frequently as *Salmonella*, it is clear that they encode many mechanisms to withstand osmotic and desiccation stress and can exhibit long term survival in challenging environments right across the farm to fork chain. Of concern also is the observation that desiccation may not lead to *E. coli* cell death, but instead entry to a VBNC state (Scherber et al., 2009). For a pathogen with an extremely low infectious dose and the high severity of the illness it can cause, it is important to be cognisant of its tolerance to low a_w and desiccation and therefore its potential to survive preservation methods or survive long-term as a contaminant within the food processing environment.

4. *Cronobacter* spp.

Cronobacter species are Gram-negative, rod-shaped, non-spore-forming motile bacteria of the family *Enterobacteriaceae*. The genus *Cronobacter* – as proposed in 2008 – currently consists of seven species according to the “List of prokaryotic names with standing in nomenclature” (<http://www.bacterio.net/allnamesac.html>, viewed, October 20th 2015) and encompasses organisms that have previously been identified as *Enterobacter sakazakii* (Iversen et al., 2008; Joseph et al., 2012). The extension of the genus *Cronobacter* by three non-pathogenic *Enterobacter* species (*E. pulveris*, *E. helveticus* and *E. turicensis*), as proposed by Brady et al. (2013) was withdrawn as the genus membership was experimentally disproved in the study by Stephan et al. (2014). These organisms are regarded as opportunistic pathogens linked with life-threatening infections, particularly in premature, low-birth weight (<2500 g) or immuno-compromised neonates and infants less than 4 weeks of age (FAO/WHO, 2008). Clinical symptoms include necrotising enterocolitis (NEC), bacteremia and meningitis, with case fatality rates ranging between 40 and 80% being reported (Bowen and Braden, 2006; Friedemann, 2009). Since the first case was documented in 1958 there have been around 120 documented cases worldwide, and at least 27 deaths (to July 2008) (FAO/WHO, 2008).

Although these ubiquitous organisms have been isolated from a range of food and environmental sources, the bulk of research has concentrated on the presence of these organisms in dairy-based powdered infant formulae (PIF), notably due to the fact that contaminated PIF has been epidemiologically linked with infections and reported illness (Bowen and Braden, 2006). In 50–80% of cases, PIF is both the vehicle and source (direct or indirect) of illness (FAO/WHO, 2004). However, not all infants with infections have been exposed to PIF, suggesting another environmental source.

Members of the genus *Cronobacter* are reported to exhibit greater osmotic and desiccation tolerance than bacteria such as *E. coli* and *Salmonella* (Breeuwer et al., 2003). This section focuses on the current knowledge on the mechanisms used by *Cronobacter* in order to survive under low a_w conditions and the resulting impact of this feature for the milk powder producing industry.

Unlike commercially available ready to eat liquid formula, dried infant formula milk powders are not sterile and are subject to stringent hygiene controls and microbiological criteria in their manufacture. Current international microbiological standards require *Cronobacter* spp. to be absent in 30 samples of 10 g (Codex Alimentarius Commission, 2008; FAO/WHO, 2006). There is general agreement that standard pasteurisation practices are effective for the inactivation of *Cronobacter* spp. and that contamination occurs post-pasteurisation (Breeuwer et al., 2003; Edelson-Mammel and Buchanan, 2004). Studies that focused

on the possible entry routes, the routes of transmission and the persistence of these pathogens in PIF processing facilities and/or final products provide evidence that contamination of the final products may take place at the drying or filling stages of production, via the addition of potentially contaminated heat labile ingredients including starches, proteins, lecithin, gums and/or via the production environment (e.g. organisms attached to dust particles) (Iversen et al., 2009; Mullane et al., 2007b; Mullane et al., 2008). The reported prevalence of *Cronobacter* spp. in surveys of commercially available PIF appears to be gradually decreasing from estimates of 14% in 1988 (Muytjens et al., 1988) and 6.7% in 1997 (Nazarowec-White and Farber, 1997), to 2.5% in 2001 (Heuvelink et al., 2002). Recent estimates indicate that although small reductions may still be possible, the prevalence appears to be stabilising at 2–2.5% (Iversen and Forsythe, 2004; Iversen et al., 2008; Mullane et al., 2007a). Additionally, the prevalence of *Cronobacter* spp. infections in adults is increased in the elderly who are immunocompromised, and may use protein supplements as part of their diet (FAO/WHO, 2008). The reported levels of contamination, however, are low ranging from 0.36 to 66.0 CFU/100 g, thereby never exceeding levels >1 CFU/g (Muytjens et al., 1988; Osaili and Forsythe, 2009; Simmons et al., 1989). It is generally considered that multiplication prior to consumption is required to cause illness. Current knowledge suggests that <3 cfu/100 g in PIF, followed by multiplication after reconstitution can lead to infection (FAO/WHO, 2004).

A wide variety of *Enterobacteriaceae* have been isolated from PIF, including *Citrobacter koseri*, *S. enterica*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pantoea agglomerans*, *Enterobacter cloacae*, and *Escherichia vulneris* (FAO/WHO, 2004; FAO/WHO, 2006). However, when compared with other members of the *Enterobacteriaceae* family, *Cronobacter* spp. are reported to be more adapted to dry stress. Lin and Beuchat (2007) investigated the effects of water availability and temperature on *Cronobacter* spp. recovered from infant cereal over a 12-month period. These data showed that an initial inoculum of 2 CFU/g could survive in infant cereals for up to 12 months over a 0.30–0.83 a_w range. In a study by Barron and Forsythe (2007) the long-term persistence of 10 *Cronobacter* strains and 17 strains of other *Enterobacteriaceae* in PIF up to 2.5 years was investigated. *Enterobacteriaceae* could be divided into four groups with respect to their long term survival in the desiccated state and only capsulated strains of *C. sakazakii* were recoverable after this extended period of time.

Physiological analysis previously linked desiccation resistance of *C. sakazakii* with trehalose accumulation. Breeuwer et al. (2003) demonstrated that the trehalose concentration in dried stationary phase *C. sakazakii* cells increased more than fivefold. However, this observation has, up to now, not been further investigated. In a study by Feeney and Sleator (2011), a comparative genomic approach was applied to investigate the distinctive ability of this pathogen to survive and persist in low a_w conditions. Homologues of all principal components of the relevant osmotic stress response mechanisms (i.e. accumulation of K⁺ and glutamate and uptake/biosynthesis of compatible solutes) described previously in *E. coli* and *Salmonella* were identified in the *C. sakazakii* BAA-894 genome. However, one key difference was the observation that *C. sakazakii* contains multiple copies of certain osmotolerance loci, including seven copies of the *E. coli* ProP homologue and two copies of OpuC, a multi-component carnitine uptake system described in *L. monocytogenes* which has also been found to transport compatible solutes such as glycine betaine, proline, ectoine and choline.

A study by Riedel and Lehner (2007) used a comparative proteomic approach to identify differentially expressed proteins playing a key role in osmotic stress response in *C. sakazakii* during application of two types of osmotic stress (physical desiccation and growth in hyperosmotic 1 M NaCl media). The protein pattern of NaCl-grown cultures demonstrated a general down-regulation of central metabolic pathways, whereas adaptation of (non-growing) cells in a desiccated state represented an accumulation of proteins that serve some structural or protective role. However, two common features – the shutdown of the motility

apparatus and the formation of filaments – were observed in response to both types of osmotic stresses applied.

In a recent study [Alvarez-Ordóñez et al. \(2014\)](#) identified several genes involved in the desiccation tolerance of *Cronobacter*. The Cpx system, an envelope stress response regulator, and the sigma factors RpoN and RpoS seem to be the main signals regulating the bacterial response to hyperosmotic conditions. Among the other identified genes, only *dnaK* and *dnaJ*, encoding two molecular chaperones, were important for *C. sakazakii* survival under desiccation. Furthermore, all *Cronobacter* contain genes for β -carotene production which is believed to protect bacteria not just from harmful oxygen radicals but also in the tolerance to physical desiccation ([Johler et al., 2010](#); [Joseph et al., 2012](#)).

Filament formation has been described for several organisms in response to a number of stresses including *E. coli* and *S. Enteritidis* at low temperature ([Phillips et al., 1998](#); [Shaw, 1968](#)) and under high osmotic stress conditions ([Mattick et al., 2000b](#)). Filament formation may occur due to the inhibition of cell division proteins as a result of osmotic stress. As a matter of fact a down regulation of the septum site-determining protein MinD was observed during both types of osmotic stresses applied to *Cronobacter* ([Riedel and Lehner, 2007](#)), and the presence of this phenotype was confirmed in hyper osmotically grown cells. Thus, it should be emphasised that filament formation may also take place in low a_w environments, such as PIF. The formation of filaments leads to an increase in the overall biomass, but without any elevation in cell numbers. Following an increase in the a_w of the PIF (e.g. rehydration), septation could resume and rapidly result in a large number of viable *Cronobacter* spp. cells, which could cause infection after consumption. Moreover, if bacterial filamentation occurs within a food product it could lead to an underestimation of the potential cell numbers present, as the filaments will not reflect the actual CFU when using conventional microbiological enumeration techniques. For *Salmonella*, it has been shown that formation of filaments prior to entrance into a dried state may lead to increased desiccation tolerance in comparison to non-filamentous cells on a stainless steel surface ([Stackhouse et al., 2012](#)). Thus, the transition into a filamentous stage may be an important survival strategy of the bacteria during milk powder processing.

Cronobacter has been known to produce various types of extracellular components, such as cellulose ([Grimm et al., 2008](#); [Lehner et al., 2005](#); [Zogaj et al., 2003](#)), colanic acid ([Scheepe-Leberkuhne and Wagner, 1986](#)) and a fucose-containing, viscous, gum like polysaccharide which was originally intended to be used as a thickener in food industry ([Harris and Oriel, 1989](#)). Colanic acid and encapsulation may contribute to adherence to some surfaces but may also contribute to resistance to dry stress. [Scheepe-Leberkuhne and Wagner \(1986\)](#) considered that colanic acid may be a significant factor contributing to biofilm formation and increased resistance to environmental stresses such as desiccation, heat, and pH in *Cronobacter* spp. One study demonstrated that an encapsulated strain of *Cronobacter* produced biofilms of a higher cell density than those produced by a nonencapsulated strain ([Iversen et al., 2004](#)). The finding that all *Cronobacter* strains that were recovered in the long term study by [Barron and Forsythe \(2007\)](#) were encapsulated provides evidence that this feature plays an important role in the survival of these organisms in low a_w environments.

A phenomenon called 'colony phase variation' has been known for a long time in *Cronobacter* spp. ([Farmer et al., 1980](#)). It describes the observation that isolates may exhibit two types of colony morphologies – dry/rugose or smooth. It has been suggested that colonies expressing the rugose phenotype contain cellulose as an extracellular polysaccharide ([Zogaj et al., 2003](#)). Various studies using other enteric organisms including *Salmonella* have shown that strains expressing the rugose phenotype impart a resistance to desiccation and antimicrobial agents such as hypochlorite; an increased ability to form biofilm and the reversible rugose to smooth colony phase variation capability ([Anriany et al., 2006](#)).

Since 2010, when whole genome sequencing efforts of *Cronobacter* species commenced one of the projects focused on a *Cronobacter*

sakazakii isolate SP291 found to persist in a PIF production facility and its genome was compared to the previously sequenced reference *C. sakazakii* BAA894, which is also of PIF origin ([Yan et al., 2013](#)). Annotation of the genome suggested that *C. sakazakii* SP291 contained a repertoire of genes that could function to aid survival under stressed conditions. Besides the loci involved in osmotolerance already assessed in the study by [Feeney and Sleator \(2011\)](#), several other genes linked to osmotic stress conditions were identified in the genome of *C. sakazakii* SP291. However, the role of one or several of these factors in osmotolerance will have to be investigated further.

The question of the features which contribute to the capability of *Cronobacter* spp. to out survive other major foodborne pathogens such as *Salmonella* in PIF is of eminent importance for the dairy industry in particular. The mechanisms used by this pathogen to survive long-term in low a_w products and production environments are only beginning to be described. These survival strategies may include, but may not be exclusive to, the accumulation of osmoprotectant molecules, filamentation, capsule production, as well as biofilm formation, including the production of various extracellular polysaccharides. Several studies indicate that biofilm or surface dried-associated phenotypes may contribute to the persistence of *Cronobacter* in the production environment. This finding clearly supports the need to continuously monitor *Cronobacter* species in the production environment and also to identify those isolates that persist. Further research will be necessary in order to understand the molecular mechanisms associated with such characteristics, which may be helpful as a means of controlling them.

5. *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, non-sporeforming foodborne pathogen, which is cold tolerant, widespread in the environment, and has the capability to grow under harsh conditions, including at elevated salt levels (up to 14%). *Listeria* has been known to persist in the food production environment for prolonged periods, despite regular sanitation ([Keto-Timonen et al., 2007](#)). Although outbreaks are relatively rare, the persistence, transfer and potential for later outgrowth in foods ([Hansen and Vogel, 2011](#); [Keskinen et al., 2008](#)) present a severe problem for the food industry due to the serious nature of listeriosis, and the vast economic consequence of product withdrawals and export problems. Many attempts have been made to identify specific characteristics in strains defined as persistent. While there is no doubt that strains generally differ in many aspects related to, for example, virulence ([Van Stelten et al., 2010](#)) or adhesion to surfaces ([Borucki et al., 2003](#); [Szlavik et al., 2012](#)), no clear picture has until now emerged regarding specific characteristics of putative persisting strains versus non-persisting strains. This has been discussed in recent reviews including [Carpentier and Cerf \(2011\)](#) and [Ferreira et al. \(2014\)](#). Once introduced in a suitable growth niche, *L. monocytogenes* strains can be expected to colonise it.

Most bacteria, including *L. monocytogenes*, have evolved to cope with increased osmolarity by the intracellular accumulation of compatible solutes and by adaptive cell envelope and proteome modifications ([Csonka, 1989](#); [Sleator and Hill, 2002](#)). In *L. monocytogenes*, a number of proteins have been shown to be involved in salt stress response ([Sleator et al., 2003](#)). This includes transporters for the uptake of compatible solutes, proteins for cell wall modification, regulatory proteins, as well as general stress proteins. Currently known and functionally characterised osmotic stress adaptation proteins and systems in *L. monocytogenes* are listed in [Table 3](#).

The main osmotic stress response mechanism in *L. monocytogenes* is the accumulation of compatible solutes ([Sleator and Hill, 2002](#); [Sleator et al., 2003](#)). The major osmoprotectants glycine betaine and carnitine cannot be synthesised de novo by *L. monocytogenes* and must therefore be taken up from the environment. Glycine betaine accumulation is mediated by BetL and Gbu ([Angelidis and Smith, 2003](#); [Gerhardt et al.,](#)

Table 3
Overview of *L. monocytogenes* osmotic stress response systems.

Stress response system	Function	Reference
BetL	Glycine betaine transporter	Gerhardt et al. (1996)
Gbu	Glycine betaine transporter	Ko and Smith (1999)
OpuC	Carnitine transporter	Fraser et al. (2000), Verheul et al. (1995)
KdpED	Kdp ATPase, two component system for K ⁺ uptake	Brondsted et al. (2003), Kallipolitis and Ingmer (2001)
ProBA	Proline biosynthesis	Sleator et al. (2001)
OppA	Oligopeptide transporter	Borezee et al. (2000)
DtpT	Oligopeptide transporter	Wouters et al. (2005)
TreA	Phosphotrehalase	Ells et al. (2011)
Lmo0501	Transcriptional regulator	Michel et al. (2011)
Lmo1078	UDP-glucose phosphorylase	Chassaing and Auvray (2007))
Lmo2085	LPXTG peptidoglycan bound protein	Utratna et al. (2011)
HtrA	General stress response serine protease	Wonderling et al. (2004)
ClpC	General stress response protein ATPase	Rouquette et al. (1998)
ClpP	General stress response protease	Gaillot et al. (2000)
CspABD	Cold shock proteins	Schmid et al. (2009)
RelA	(p)ppGpp synthetase	Okada et al. (2002)
Ctc	General stress protein, L25 family of ribosomal proteins	Gardan et al. (2003b)
LisRK	Two component regulatory system	Sleator et al. (2005)
lap	Invasion associated protein	Burall et al. (2012)
MrpABCDEFG	Na ⁺ /H ⁺ antiporter	Bergholz et al. (2012)
σ ^B	Alternative sigma factor σ ^B	Chaturongakul et al. (2008), O'Byrne and Karatzas (2008)

1996; Ko and Smith, 1999), whereas carnitine is taken up by OpuC (Fraser et al., 2000; Verheul et al., 1995). In addition, the Kdp system allows K⁺ import, facilitating a rapid response to osmotic stress (Brondsted et al., 2003; Kallipolitis and Ingmer, 2001; Sleator and Hill, 2002). The compatible solute trehalose is important for the survival of many organisms under adverse environmental conditions (Elbein et al., 2003). A recent study characterised the phosphotrehalase gene *treA* (*lmo1254*) and indicated the role of the trehalose metabolism in stress resistance (Ells and Truelstrup Hansen, 2011). Proline is another main osmoprotectant (Csonka, 1989; Sleator and Hill, 2002). In *L. monocytogenes*, proline biosynthesis by ProBA is increased under osmotic stress (Sleator et al., 2001). An alternative source of peptides which can subsequently be hydrolysed to osmoprotective amino acids, such as proline, are the Opp and DtpT oligopeptide transport systems (Borezee et al., 2000; Wouters et al., 2005). Two genes associated with cell envelope modification in response to osmotic stress have been identified recently: *lmo2085*, a putative peptidoglycan bound protein, and *lmo1078*, a putative UDP-glucose phosphorylase catalysing the formation of UDP-glucose, a precursor of membrane glycolipids and cell wall teichoic acids (Chassaing and Auvray, 2007; Utratna et al., 2011). A recent study using transposon mutagenesis identified the *lscC* gene to be involved in high salt tolerance (Burall et al., 2015); however, the function of the *lscC* gene, is currently unknown. The transcriptional regulator *lmo0501* is involved in osmotic stress response; a *lmo0501* deletion mutant showed reduced growth under salt stress conditions (Michel et al., 2011). In addition, general stress response proteins including the serine protease HtrA, which may play a role in degrading misfolded proteins, ClpC (an ATPase), and ClpP (a protease) are essential for osmotic stress adaptation in *L. monocytogenes* (Gaillot et al., 2000; Rouquette et al., 1998; Wonderling et al., 2004). HtrA is under the control of the LisRK two component regulatory system, which is important for osmosensing and osmoregulation (Sleator and Hill, 2005). Furthermore, RelA, a guanosine tetra/penta-phosphate [(p)ppGpp] synthetase is involved in adaptation to various environmental conditions (Braeken et al., 2006), and has been shown to be essential for osmotic

stress response in *L. monocytogenes* (Okada et al., 2002). The authors suggested that an appropriate intracellular concentration of (p)ppGpp is essential for osmotolerance; this mechanism is distinct from that for the accumulation of compatible solutes. Similarly, Ctc, belonging to the L25 family of ribosomal proteins, is involved in osmotic stress tolerance in the absence of compatible solutes, although the function of Ctc proteins is still unclear (Gardan et al., 2003b). A recent transcriptomic study revealed evidence for an important role of the MrpABCDEFG sodium/proton antiporter in adaptation to salt stress in *L. monocytogenes* similar to results from other *Firmicutes* (Bergholz et al., 2012). Interestingly, the *iap* gene also plays an important role in cold and salt adaptation, as revealed by transposon mutation and gene expression studies (Burall et al., 2012; Olesen et al., 2009). Many of the aforementioned (experimentally characterised) genes are under the control of the alternative sigma factor σ^B, which controls a plethora of genes involved e.g. in stress tolerance, metabolism, transport, cell envelope, and virulence (Chaturongakul et al., 2008; O'Byrne and Karatzas, 2008).

In addition to functionally characterised genes, a number of studies performed global analyses to identify additional genes involved in osmotic stress tolerance in *L. monocytogenes* including proteomic (Abram et al., 2008; Duche et al., 2002; Melo et al., 2013a, 2013b; Pittman et al., 2014), transcriptomic (Bae et al., 2012; Bergholz et al., 2012; Durack et al., 2013; Ribeiro et al., 2014), as well as transposon-based mutational studies (Burall et al., 2012; Gardan et al., 2003a). A large number of candidate genes have been identified in these studies; however, functional characterisation will be needed in the future to clarify the contribution of these genes to osmotic stress tolerance in *L. monocytogenes*. Several studies have shown that exposure of *L. monocytogenes* to sublethal stresses can induce the development of stress-conditioned strains, which show higher tolerance to increased levels of the same or different stresses. A cross-protection between osmotic and cold stress has been shown for cold shock proteins CspABD (Schmid et al., 2009), *lap* (Burall et al., 2012), *lmo0501* (Michel et al., 2011), and *lmo1078* (Chassaing and Auvray, 2007).

Part of the ability of pathogens to survive in foods and food processing plants is associated with the ability to withstand matrix and osmotic stress caused by desiccation or external osmolytes. *L. monocytogenes* has been frequently isolated from environments which are intermittently exposed to low humidity such as equipment surfaces (Carpentier and Cerf, 2011) and soil (Dowe et al., 1997; Piveteau et al., 2011). More sporadically, positive samples from food products with low *a_w* such as dry cured sausages (Farber et al., 1988), nuts, dried milk and dried fish have been reported. There is limited information on desiccation tolerance compared to other foodborne pathogens and results may vary according to the experimental set-up and strains employed. In a comparative study investigating desiccation tolerance at 23 °C on walnuts inoculated with cocktails of *Salmonella*, *E. coli* and *L. monocytogenes*, respectively, the latter, consisting of four serotype 4b strains and one serotype 1/2a strain, declined more rapidly (Blessington et al., 2012). This is in contrast to a single strain study where a *Salmonella*, an *E. coli* O157:H7 and a *L. monocytogenes* strain were dried in chicken manure, and where the two Gram negative bacteria both decreased significantly faster than the *Listeria* (Himathongkham and Riemann, 1999).

Little is known of the mechanisms behind desiccation tolerance. Dreux et al. (2008) found that the survival of *L. monocytogenes* L028 (serotype 1/2c) inoculated into microtitre wells at reduced humidity (RH%: 60 ± 13), correlated with the amount of glycine betaine added to the suspension fluid. On parsley leaves, only large (>25 mmol/L) concentrations of glycine betaine enhanced initial survival. Remarkably, however, mutants without uptake systems for glycine betaine exhibited the same increase in survival as the wild type when inoculated with high amounts of added glycine betaine, indicating that intracellular accumulation of glycine betaine is of little importance under these circumstances. When wild type and glycine betaine uptake deficient

mutants were pre-incubated with 3% salt and glycine betaine, they both exhibited slight increases in survival. A significant protective effect of salt (5%) has also been reported by others (Hingston et al., 2013; Vogel et al., 2010), and in some cases indications of better survival after salt adaptation have been reported (Hansen and Vogel, 2011), although the mechanism has not been elucidated. Enhanced survival in the presence of different types of food contamination has been observed in several studies (Palumbo and Williams, 1990), and high amounts of lard have been shown to especially affect the initial drop in viability (Hingston et al., 2013). *Listeria* does not naturally accumulate the potent osmoprotectant trehalose, but experiments employing a trehalose fed $\Delta TreA$ deletion mutant unable to metabolise trehalose further, showed increased desiccation resistance (Ells and Truelstrup Hansen, 2011). Higher temperature during dry storage results in higher die-off, whether this is in low a_w foods or on soiled surfaces at low relative humidity (De Roin et al., 2003; Nissen and Holck, 1998; Palumbo and Williams, 1990). Using steel coupons Hingston et al. (2013) showed, that desiccation kinetics were independent of the cell surface densities upon surface inoculation. However, if allowed to form biofilm for 48 h, only the higher contamination levels formed mature biofilms, resulting in a pronounced protective effect against desiccation. In summary, a number of extracellular factors have been identified enhancing the desiccation tolerance, although there is still very limited understanding of the role of physiological changes in desiccation survival in *L. monocytogenes*.

6. *Campylobacter* spp.

As an obligate microaerophilic and capnophilic microorganism, the human pathogen *Campylobacter* spp. is well adapted to the intestinal tract of warm blooded animals. With an optimal growth at 42 °C under microaerobic atmospheres, its main reservoir is the bird gut. Poultry, and to a lesser extent pork and cattle, represent the main risk of *Campylobacter* transmission to humans. Human infection by *Campylobacter* occurs most frequently through contaminated poultry food products or foodstuff cross contaminated directly from poultry products or indirectly throughout food transforming processes (FAO/WHO, 2009; Guyard-Nicodeme et al., 2013). The two main pathogenic species include *C. jejuni* and *C. coli*, with a higher prevalence of *C. jejuni* (Wassenaar and Newell, 2006). This asaccharolytic micro-organism is characterised by the absence of a functional glycolysis system. Thus, the presence of a high level of amino acids in the gut constitutes a rich carbon source for *Campylobacter* growth through gluconeogenesis. In addition, with the presence of a specific genomic island, certain strains of *C. jejuni* can challenge other enteric bacteria by uptaking and utilising L-fucose, a carbohydrate metabolism which has not yet been described in the human gut microbiota (Stahl et al., 2011). With a marked increase in incidence, *Campylobacter* has become the leading cause of bacterial foodborne diseases in Europe (EFSA, 2010, 2012, 2013). To transfer from farm animals to humans it has, therefore, to face the challenges of food environments and to survive conditions outside its main hosts.

To multiply in its host niche and in the human gut, *Campylobacter* has to adapt to various osmotic concentrations. The osmolarity in the chicken duodenum and caeca at 42 °C is 0.7 and 0.9 osmol/L, respectively, while it is around 0.3 osmol/L in the human intestine at 37 °C (Cameron et al., 2012; Reezal et al., 1998). As a foodborne pathogen, it also has to adapt to osmotic concentrations in food products which can vary widely according to the food processes, the contaminated food and the food conservation method. The osmotic concentration threshold for *C. jejuni* growth at 42 °C has been determined between 0.143 to 0.146 osmol/L under optimal growth conditions (microaerobic atmosphere) (Reezal et al., 1998).

At hypo-osmotic concentrations, *C. jejuni* showed a lower ability to resist at refrigeration temperatures. In addition, low osmolarities favour coccoid shaped cells while the spiral or rodlike shape is commonly

described in optimal growth conditions for *Campylobacter* spp. Under low osmolarities, the coccoid shape is correlated to an increase of VBNC cells (Reezal et al., 1998). Coccoid and VBNC cells are the signature of adaptation and protection of *Campylobacter* under stressed conditions (Beumer et al., 1992; Hazeleger et al., 1994; Park, 2002). At refrigeration temperatures, *C. jejuni* cells can recover from medium osmolarities, while osmolarities below 0.130 osmol/L are lethal for this pathogen. This finding was described using both osmolyte-controlled media or complex rich media (Reezal et al., 1998).

At hyperosmotic concentrations, *C. jejuni* can maintain growth until 0.171 osmol/L. In combination with temperature, the hyperosmotic medium enhances the rate of *C. jejuni* death at 25 °C, although it is strain dependent. However, at refrigeration temperatures, *C. jejuni* can survive and recover from higher osmotic concentrations (2.22 osmol/L) (Doyle and Roman, 1982). Morphological changes under hyperosmotic stress in favour of septum defects leading to cell elongation have been observed (Cameron et al., 2012; Doyle and Roman, 1982). In addition, cross protection can occur, as suggested by Stintzi (2003), after a cold shock that enhances levels of proteins involved in oxidative stress response and a metabolic activity rate in *C. jejuni* higher at 4 °C than at 20 °C.

In general, *Campylobacter* is described as sensitive to desiccation compared to other foodborne pathogens (Fernandez et al., 1985). The minimum a_w for growing *Campylobacter* has been defined at 0.987 with an optimum at 0.997. At the scale of chicken broilers, a litter a_w at 0.5, in humidity-controlled pens, reduced *Campylobacter* colonisation rates as compared to a litter a_w at 0.795 and subsequently reduced the contamination of chicks (Line, 2006).

Two proteins involved in a non-amino acid specific ABC transporter, PaqP (pathogenesis-associated glutamine ABC transporter permease) and PaqQ (pathogenesis-associated glutamine ATPase), were found to be involved in osmoregulation in *C. jejuni* 81-176, a highly pathogenic strain (Korlath et al., 1985). Deletion of PaqP or PaqQ impaired the uptake of glutamine, and to a lesser extent glutamate, cysteine and aspartate. Concomitantly, the PaqP defective mutant tolerated hyperosmotic stress better than the parental strain and the PaqQ deficient mutant was more sensitive (Lin et al., 2009). The uptake of amino acids, which is the only carbon source in *Campylobacter* is therefore correlated to osmoregulation. The balance of amino acid availability in the cells affects *Campylobacter* survival which could be compensated by an adaptive response requiring osmoregulation.

Amino acid uptake is also controlled by genes regulated by ppGpp (guanosine tetraphosphate) which is formed from GTP (guanosine triphosphate) in cells (Paul et al., 2005). Overall, the *C. jejuni* genome, as with many other bacteria, contains two conserved polyphosphate kinases (PPK1 and PPK2) (Zhang et al., 2002). The PPK1 is involved in anabolism of polyP and PPK2 is more oriented to the generation of GTP. The survival of $\Delta ppk1$ *C. jejuni* was lower in hyperosmotic stress which correlated to a poly-P accumulation defect, confirming the biological role of PPK1 in polyP anabolism in this species (Candon et al., 2007). A dramatic decrease of resistance to hyperosmotic stress was also observed in a PPK2 deleted *C. jejuni* mutant (Gangaiah et al., 2010). However, it was not concomitant with a variation of ppGpp level in this mutant. This could be explained by the presence of genes in *C. jejuni* that could alternatively ensure the ppGpp level, such as that of the putative nucleoside diphosphate kinase (NDK) and SpoT, a bifunctional ppGpp synthase/hydrolase (Gangaiah et al., 2010). In the *C. jejuni* $\Delta ppk2$ mutant, poly-P-dependent GTP generation was defective, confirming its function in GTP generation in this microorganism (Gangaiah et al., 2010). The inorganic phosphate poly P constitutes an energy reservoir in the cells. It is also related to virulence and stress responses in pathogenic bacteria (Kornberg et al., 1999). Consequently, in *C. jejuni*, the role of PPK1 and PPK2 is important in poly P-accumulation and in GTP generation, respectively, in response to hyperosmotic stress, but it is not mediated by ppGpp to maintain the inorganic poly P level in cells.

In many bacteria, conserved sigma factors promote the attachment of RNA polymerase to specific initiation sites. They are involved in many cell processes including stationary phase elicitation, virulence and stress defence. Interestingly, in *C. jejuni* genomes, only three sigma factors have been described (RpoD, FliA and RpoN) (Gundogdu et al., 2007; Parkill et al., 2000). The σ^{38} factor (RpoS) which is a crucial regulator in osmotically regulated genes in most Gram-negative bacteria (Muffler et al., 1996) has not been identified in *Campylobacter* spp. However, the pathogenic strain 81-176 with a *rpoN* (σ^{54}) mutation exhibited significant impairment of growth capability under hyper-osmolarity (0.274 osmol./L) indicating the involvement of this sigma factor in *C. jejuni* osmoregulation. The elongation cell feature is even enhanced in the $\Delta RpoN$ mutant under hyper-osmotic stress (Okada et al., 2006). A major role of RpoN in foodborne pathogens' osmotolerance has also been described previously (Hwang et al., 2011), while σ^B is more commonly detected as the sigma factor in response to general stresses in Gram-positive bacteria (Van Schaik and Abee, 2005).

From these observations, *C. jejuni* is not the most osmo- and desiccation-tolerant among foodborne pathogens. Although no endogenous osmoprotectant molecule has been described up to now in *Campylobacter* spp. and its genome lacks enzymes to generate trehalose, glycine betaine or proline, alternative strategies are used by *Campylobacter*, likely a cross protection against stresses, particularly at refrigeration temperatures. It is important to point out that the osmo- or desiccation-adaptation of *C. jejuni* to the food environment is different for non-growing cells and growing cells. As non-growing cells in food products, *Campylobacter* can enhance its resistance power as dormant cells. For instance, *Campylobacter* has the ability to better resist to hyperosmotic stresses at refrigeration temperatures (Doyle and Roman, 1982). However, many experiments are conducted on growing cells, although *Campylobacter* is a survivor rather than a growing bacterium in food products. Its genome apparently possesses alternative strategies to limit the impact of osmotic shock for its survival throughout the farm to fork food chain. A second observation concerns the high variability potential of the species, as 25 high variable regions have been described in the genome of *C. jejuni* (Parkill et al., 2000), especially within loci involved in the synthesis of surface components containing sugar residues such as lipooligosaccharides, capsular components and glycosylation processes. This unique feature is correlated to the high genetic diversity of strains among *Campylobacter* species. This intra-species genetic fitness, and the resulting bacterial surface properties, might play an important role in the strain adaptation capability to osmotic and desiccation stresses, as the bacterial membrane is the first defence against environmental stresses. However, in general, the studies performed on the two most pathogenic species of *Campylobacter* utilised a very low representation of the strain variability. Consequently, further analyses should focus on a better representation of the strain variability to determine and to better characterise the response of pathogenic *Campylobacter* to food environmental stresses such as osmotic and desiccation stress.

7. Concluding comments

This review has clearly demonstrated that foodborne pathogens have evolved a number of survival mechanisms to address the challenges imposed on them by food processing and preservation technologies. These studies highlight the unique characteristics of low-moisture foods which make them a special challenge for the food industry. It is apparent that among foodborne pathogens there is no unique and single response to osmotic stress and desiccation. The main described responses to these stresses include the regulation and accumulation of osmoprotectants, cross protection in response to other food stresses and the contribution of EPS or membranous components such as colanic acid or disaccharides. New perspectives to understand the underlying mechanisms should encompass the role of fatty acids, ribosomal protein degradation and regulation of the intracellular ppGpp pool. Many

studies have been undertaken using single, or very few, reference strains which are not necessarily representative of a species in general. Greater consideration needs to be given to undertaking carefully designed experiments including a broad range of strains from relevant ecological niches to ensure the results obtained are more representative of the species as a whole. This will enable more accurate risk assessment to be undertaken.

Considering the tolerance of many pathogens to desiccation, and their ability to survive for extended time periods in food processing environments, the food industry must further consider the safe manufacture of low a_w foods. The hygienic design of the manufacturing infrastructure, and in particular the food processing equipment, are well established (EHEDG, 2007, 2010, 2014) but are fundamental in reducing pathogen harbourage sites and allowing access to cleaning chemicals to facilitate surface decontamination. Cleaning and disinfection programmes can be modified to focus more attention on the disinfection stage, as the majority of sanitation programmes for low a_w foods are predominantly about physical removal of soils via dry cleaning techniques, particularly those such as ozone, hydrogen peroxide and UV light that would not introduce moisture. What would be most useful, however, is how the food manufacturing environment could be manipulated in terms of e.g. humidity, temperature, frequency and duration of any wet cleaning activities, or perhaps pH extremes via acidic and alkaline detergents, to disrupt the persistence of desiccation tolerant pathogens.

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